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Detecting Novel Effects of Exercise or AMPK Activation in Human Skeletal Muscle

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Detecting Novel Effects of Exercise or AMPK Activation in Human Skeletal Muscle
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Cardiovascular and metabolic disorders are among the main causes of death today. Regular exercise can prevent and treat these chronic diseases. A molecule at the center of exercise adaptations in skeletal muscle is adenosine monophosphate-activated protein kinase (AMPK). Rapid energy turnover in cells, such as during contraction in skeletal muscle, activates AMPK. The activation of AMPK leads to inhibition of anabolic processes that consume energy and upregulation of catabolic processes that generate energy. AMPK activation increases glucose uptake into peripheral tissues. Even insulin-resistant individuals, including type 2 diabetes patients, retain the blood-glucose lowering effect of AMPK activation.

There is a need to better understand how exercise provides protective benefits, and how AMPK functions at the cellular level. This thesis consists of three research papers wherein exercise and AMPK activation were used as experimental models to identify novel effects of these signals in human skeletal muscle.

The first paper explores how fasting between consecutive bouts of exercise from one day to the next enhances the adaptive response. Overlapping fasting with exercise increases AMPK signaling and expression of genes that regulate fat oxidation. In addition, the combination of exercise and fasting elicits changes in DNA promoter methylation. Fasting overnight between an evening and morning training session enhances the adaptive benefits of exercise.

The second paper investigated if AMPK activation and insulin signaling affect the focal adhesion kinase (FAK) in a differential manner in human skeletal muscle. FAK is a necessary component for insulin and growth signaling in non-human skeletal muscle models, and cancer researchers are exploring FAK inhibitors as cancer therapeutics. The new research provided herein demonstrates that insulin does not activate FAK in human skeletal muscle. However, AMPK does inhibit FAK. Furthermore, siRNA-mediated silencing of FAK in cells increases lipid oxidation. AMPK inhibits FAK, and FAK affects substrate utilization in skeletal muscle cells.

In the final paper, a bioinformatic analysis identified genes that are regulated by AMPK, including the gene for ganglioside-induced differentiation-associated protein 1 (*GDAP1*). *GDAP1* regulates mitochondrial function in nerve cells. Silencing *GDAP1* does not alter mitochondrial function or morphology. However, silencing *GDAP1* does reduce lipid oxidation, non-mitochondrial respiration, and alters the expression of circadian genes. AMPK regulates *GDAP1* expression, and *GDAP1* alters lipid oxidation without affecting mitochondrial function in skeletal muscle.

The key findings of the thesis are; 1) combining fasting with exercise impacts the epigenetic state in muscle and induces adaptive changes that promote lipid oxidation, 2) AMPK-mediated FAK inhibition may be a therapeutic strategy to treat cells which have a reduced capacity to oxidize lipids, and 3) *GDAP1* in skeletal muscle plays a role in modulating the core circadian clock and non-mitochondrial lipid oxidation.

SVENSKA SAMMANFATTNING

Idag dör många människor på grund av hjärt- eller metaboliksjukdomar. Dessa sjukdomar kan undvikas och behandlas genom regelbunden fysiskt träning.

En molekyl som är viktigt för att driva adaptation av skelettmuskel av träning är adenosin monofosfat-aktiverat protein kinas (AMPK). AMPK aktiveras när energinivån i en cell är låg. När AMPK aktiveras, stängs processer som kräver energi av medan processer som tillverker energi aktiveras. En viktig roll för AMPK är att den kan leda till en minskning av blodsocker, även i personer med insulinresistans så som patienter med diabetes typ 2.

Mycket forskning har genomförts om träning och hur AMPK fungerar, men det finns ett behov att bättre förstå mekinismer som ger fördelarna från träning. Denna avhandling består av tre vetenskapliga projekt där träning och AMPK aktivering användades att identifiera nya effekter av dessa signaler i mänsklig skelettmuskel.

Den första artikel i avhandlingen studerar hur fasta mellan träningstillfällen kan förbättra kroppens möjlighet att svara på träningen. AMPK-signalering samt gener som stimulera fettoxidering ökade. Metylering av DNA studerades även.

I den andra artikel studerades om AMPK-aktivering och insulinstimulering påverkar focal adhesion kinase (FAK) på olika sätt i mänsklig skelettmuskel. Även om insulinstimulering ökar FAK aktivering i andra celler, visar denna forskning att insulin inte aktiverar FAK i human skelettmuskel. Vi identifierar även att FAK hämmas när AMPK aktiveras. Slutligen, när FAK-aktivering minskat genom siRNA transfektion, är fettoxidation höjd.

I den sista artikel, identifierats nya gener som regleras av AMPK-aktivering. En gen är ganglioside-induced differentiation-associated protein 1 (*GDAP1*), en gen som underhåller mitokondriell funktion i nervceller. När *GDAP1* hämmas genom siRNA transfektion i humana skelettmuskelceller, påverkas inte mitokondriernas form eller funktion. Däremot identifierar vi en effekt på gener viktiga för reglering av dygnsrytm och icke-mitokondriell respiration.

Sammanfattningsvis, 1) att fasta mellan träningstillfällen kommer att öka effekter av träningen och påverkar epigenetiska tillstånd i muskel samt transkription och translation av gener viktiga för fettoxidering, 2) att hämma FAK genom AMPK-aktivering kan vara en strategi att behandla celler med reducerad förmåga att förbränna fett, och 3) i human skelettmuskel spelar *GDAP1* en roll i reglering av dygnsrytm och icke-mitokondriell respiration.

LIST OF SCIENTIFIC PAPERS

I. Lane SC, Camera DM, **Lassiter DG**, Areta JL, Bird SR, Yeo WK, Jeacocke NA, Krook A, Zierath JR, Burke LM, Hawley JA. Effects of sleeping with reduced carbohydrate availability on acute training responses. *J Appl Physiol (1985)*. 2015 Sep 15; 119(6): 643-55.

II. **Lassiter DG**, Nylén C, Sjögren RJO, Chibalin AV, Wallberg-Henriksson H, Näslund E, Krook A, Zierath JR. FAK tyrosine phosphorylation is regulated by AMPK and controls metabolism in human skeletal muscle. *Diabetologia*. 2018 Feb; 61(2): 424-432.

III. **Lassiter DG**, Sjögren RJO, Gabriel BM, Krook A, Zierath JR. Lipid oxidation in skeletal muscle is impaired due to *GDAP1* silencing, an AMPK-regulated gene. *Manuscript submitted to Diabetes*.

SCIENTIFIC PAPERS NOT INCLUDED IN THIS THESIS

Mudry JM, **Lassiter DG**, Nylén C, García-Calzón S, Näslund E, Krook A, Zierath JR. Insulin and Glucose Alter Death-Associated Protein Kinase 3 (DAPK3) DNA Methylation in Human Skeletal Muscle. *Diabetes*. 2017 Mar; 66(3): 651-662.

Nylén C, Aoi W, Abdelmoez A, **Lassiter DG**, Lundell L, Wallberg-Henriksoon H, Näslund E, Pillon N, Krook A. IL6 and LIF mRNA expression in skeletal muscle is regulated by AMPK and the transcription factors NFYC, ZBTB14 and SP1. *Am J Physiol Endocrinol Metab*, accepted for publication.

CONTENTS

LIST OF ABBREVIATIONS

ACC	acetyl-coenzyme A carboxylase
ADP	adenosine diphosphate
AICAR	aminoimidazole-4-carboxamide ribotide
AMP	adenosine monophosphate
AMPK	adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
ATP	adenosine triphosphate
ATPase	ATP hydrolase
BMI	body mass index
CPT1	carnitine-palmitoyl transferase 1
DUSP	dual-specificity phosphatase
ECAR	extracellular acidification rate
ECL	enhanced chemiluminescence
FABP3	fatty-acid binding protein 3
FAK	focal adhesion kinase
FBS	fetal bovine serum
GDAP1	ganglioside-induced differentiation-associated protein 1
GEO	gene expression omnibus
GLUT4	glucose transporter type 4
GWAS	genome-wide association studies
HbA_{1C}	hemoglobin A1C
HDL-C	high-density lipoprotein cholesterol
HOMA-IR	homeostasis model assessment-insulin resistance
HRP	horseradish peroxidase
LDL-C	low-density lipoprotein cholesterol
MCoA	malonyl-coenzyme A
NAD^+	nicotinamide adenine dinucleotide
OCR	oxygen consumption rate
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

PGC-1α	peroxisome proliferator-activated receptor γ coactivator 1 α
PTK2	the gene coding for FAK
PVDF	polyvinylidene difluoride
qPCR	quantitative polymerase chain reaction
RER	respiratory exchange ratio
RMA	robust multi-array averaging
RQ	respiratory quotient
RT	reverse transcription
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
RYGB	Roux-en-Y gastric bypass
SIRT1	sirtuin 1
TBC1D1	TBC domain family member 1
TBC1D4	TBC domain family member 4
TBST	tris-buffered saline with Tween-20
T2D	type 2 diabetes
VCO_2	volume of CO_2 produced during respiration
VO_2	volume of O_2 consumed during respiration

1 INTRODUCTION

1.1 EXERCISE IS MEDICINE

The leading cause of death globally in 2015 was ischemic heart disease, whereas stroke and diabetes were ranked as the second and sixth biggest killers, respectively [1]. Tightly linked to these causes of death is the increase in rates of overweight and obesity. Although the global population has not doubled since 1979, the prevalence of obesity nearly tripled by 2016, affecting over 650 million individuals [2]. The prevalence of type 2 diabetes (T2D) is also growing at a startling rate. In 2004, it was projected that one out of every 25 people will be diagnosed with diabetes by 2030 [3]. The estimate was updated in 2014 to reflect the growing problem; by 2035, one of every 10 people on the planet are projected to have diabetes [4]. While reports that rates of T2D have plateaued in the United States must be viewed positively, 10% of the US population already has the disease [5]. Simultaneously, rates of the condition continue to grow in developing countries [6-8]. Sadly, life expectancy is blunted in the presence of diabetes or obesity [9]. Metabolic disorders also lead to a reduction in quality of life [10] and an increased financial burden [11].

A myriad of treatment options exist to ameliorate the multiple burdens of obesity, cardiovascular disease, and metabolic disorder. Patients can benefit from surgery, prescription medications, altered diet, or adopting a physically active lifestyle depending on the severity of their situation. Of these options, exercise may be the most robust intervention available to enhance health, since it plays a role in the prevention and treatment in each of these conditions, as well as others.

Regular physical activity is a key component to achieve sustained weight loss [12] and weight loss, *per se*, reduces blood pressure, blood lipids, and plasma glucose in overweight individuals [13]. Additionally, regular exercise reduces visceral adipose tissue as compared to diet-induced weight loss [14]. The benefits of exercise extend far beyond weight maintenance, however.

In the context of cardiovascular health, a primary benefit of engaging in regular exercise is increased maximal cardiac output [15]. Physical activity in older adults is negatively associated with cardiovascular events [16] and patients living with coronary heart disease have a reduced risk of mortality if they are more active [17]. Compared to age-matched non-athletes, young and old athletes have higher levels of high-density lipoprotein cholesterol (HDL-C), lower levels of low-density lipoprotein cholesterol (LDL-C), and therefore a more favorable HDL-C to LDL-C ratio [18]. Cross-sectional data from women and men aged 30–54 reveal a negative correlation between physical activity and cardiometabolic risk [19]. Furthermore, exercise reduces blood pressure —the best single predictor for unfavorable cardiovascular events— in those with essential hypertension [20]. Importantly, exercise training in patients who previously suffered from heart failure is safe and improves exercise capacity [21].

Exercise, while an essential aspect of weight maintenance programs and rehabilitation after cardiovascular incidents, also ameliorates the symptoms of metabolic disorders such as T2D. Adoption of an exercise routine slows the progression to T2D [22]. With less than 12 weeks of training, and before significant changes in weight circumference are achieved, exercise leads to a reduction in fasting glucose [23]. Exercise interventions improve the homeostasis model assessment-insulin resistance (HOMA-IR) and HDL-C in patients with T2D [24]. Increased walking activity, as measured by pedometers or accelerometers, also associates with reductions in hemoglobin A1C (HbA_{1C}) in people with T2D [25]. Patients with T2D also develop enhanced heart rate variability due to regular exercise [26], which is noteworthy since these patients have reduced cardiorespiratory fitness to begin with [27].

In instances when lifestyle interventions and pharmacological treatments have insufficiently improved the health status of severely obese patients, surgery may be indicated. Roux-en-Y gastric bypass (RYGB) results in rapid improvements in body weight and insulin sensitivity, with continued improvements extending for at least 24 months [28]. RYGB even outperforms intensive lifestyle modifications in reversing T2D [29]. Nonetheless, exercise interventions extend the benefits of the surgery. Patients who are more physically active in the months after RYGB lose more weight and fat mass [30], have improved insulin sensitivity and HDL-C [31], as well as improved cardiorespiratory fitness and mitochondrial respiration [32].

Whereas an active lifestyle yields some protection against cardiovascular and metabolic disease, a sedentary lifestyle leads to a greater susceptibility to chronic diseases. Drivers of double-decker busses in London, who spent their workdays seated, had elevated heart-disease incidence and mortality as compared to the busses' conductors, who regularly walked up and down the aisles and stairs to check tickets [33]. More recently, research using pedometers and oral-glucose tolerance tests identified a 22% increased odds ratio of T2D per hour of sedentary time [34]. Physical inactivity is also correlated to some cancers [35] and depression [36]. Scientists use bed rest in research settings to induce skeletal muscle atrophy and to study the acute effects of inactivity. Notably, regular resistance exercise minimizes the detrimental effects of inactivity on skeletal muscle mass [37]. This effect of exercise is important given that there is a negative correlation between mortality and skeletal muscle mass [38]. Unfortunately, exercise interventions are not sufficient to overcome all of the harmful effects of inactivity on bone density [39], immune cell population [40], markers of liver impairments [41], or expression of genes central to an oxidative phenotype in skeletal muscle [42]. Inactivity also impairs one's ability to dispose of a lipid-rich meal, and an acute exercise bout is insufficient to recover normal postprandial lipemia [43]. Bedrest impairs insulin sensitivity [44] and even lifelong athletes develop reduced glucose tolerance after just 10 days of inactivity [45]. A sedentary lifestyle is especially harmful for older adults, since they exhibit more atrophy and reduced muscle-protein synthesis compared to younger counterparts when subjected to bed rest [46]. An inactive sedentary lifestyle leads to poor health, whereas a physically active lifestyle promotes good health.

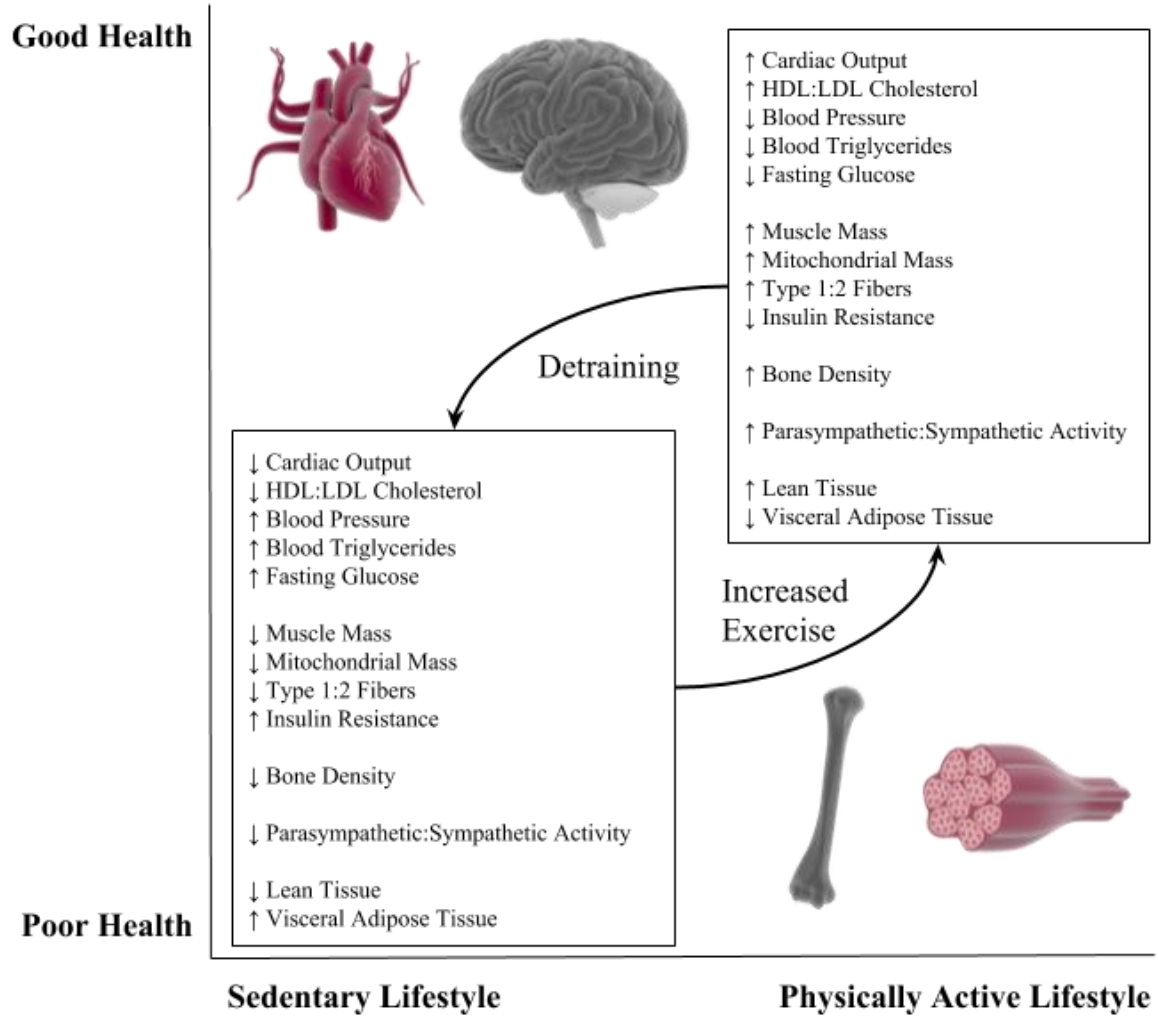

Figure 1:

The Relationship between Physical Activity and Health

Today, more people are living in countries where overnutrition is deadlier than malnutrition [2]. Thus, there is concerted effort among researchers to examine the mechanisms that lead to the positive metabolic outcomes associated with physical activity. The first scientific paper presented in this thesis explores the hypothesis that exercise and nutrition timing interact to modulate metabolic adaptations including DNA methylation. The second and third papers of the thesis focus on a key molecule that regulates energetic balance in skeletal muscle during exercise: adenosine monophosphate-activated protein kinase (AMPK). In the second paper, the hypothesis that AMPK and insulin differentially regulate a growth-promoting protein in skeletal muscle is examined. In the final paper, a bioinformatic strategy identifies a new link between AMPK and a gene that has surprising effects on skeletal muscle metabolism and circadian gene expression. Prior to describing the research methods, a review of metabolic flexibility and AMPK is warranted. To conclude the introduction, the aims of the thesis are described.

1.2 METABOLIC FLEXIBILITY: A PRIMARY ADAPTATION TO EXERCISE

One of the primary adaptations to repeated bouts of exercise is an enhanced ability of skeletal muscle to alternate between lipids and carbohydrates for meeting energy demands. An early explanation for the etiology of diabetes mellitus was that an overabundance of lipids inhibit carbohydrate oxidation [47]. The ability to adequately alternate between carbohydrate and lipid metabolism to meet energy demands is termed metabolic flexibility and an inability to switch between these substrates is referred to as metabolic inflexibility [48]. Similar to in economic theory, there are elements of supply and demand when it comes to metabolically flexibility. During periods of fasting, there is a reduced supply of carbohydrate, and metabolically flexible individuals predominantly oxidize stored lipids to sustain biological function. In the postprandial period after a meal, metabolically flexible individuals increase glucose oxidation in response to elevations in blood sugar and pancreatic insulin secretion [48]. On the demand side of the equation, metabolically flexible individuals oxidize lipids to meet resting energy requirements. However, in the transition from rest to exercise —and as exercise intensity continues to increase— metabolically flexible individuals upregulate carbohydrate oxidation, both in absolute and relative terms, to meet the heightened energy needs [49]. The workload at which an individual begins using more carbohydrates than lipids to meet energy demands is the "crossover" point [50].

Researchers can measure consumed oxygen (VO_2) and produced carbon dioxide (VCO_2) to assess which substrates individuals oxidize at a given moment in order to meet energy demands. The ratio of VCO_2 to VO_2 measured by arteriole-venous differences is the respiratory quotient (RQ). Indirect calorimetry is a less invasive technique to measure VCO_2 and VO_2 by collecting gasses at the mouth. The ratio of gases measured in this manner is the respiratory exchange ratio (RER) and it is a relatively good approximation of RQ. The full chemical combustion of a glucose molecule produces the same number of CO_2 molecules as O_2 molecules consumed. Thus, an RER of 1.0 is indicative of full reliance on carbohydrate sources for energy production. In contrast, it takes 23 molecules of O_2 to fully combust a single molecule of palmitic acid (the most abundant fatty acid in the body), producing only 16 CO_2 molecules. Accordingly, an RER of 0.7 indicates full reliance on lipid sources for energy production. A person has metabolic flexibility if RER is near 0.7 while at rest or in a fasting state (when energy supplies and demands are low) but exhibits an RER increase to near 1.0 after a meal or during intense exercise (when energy supplies or demands are high).

When RER is measured during insulin stimulation, obese individuals have reduced metabolic flexibility as compared to lean individuals [48]. Patients with T2D have reduced free fatty acid oxidation in fasting conditions as well as a reduced ability to increase glucose oxidation after a meal [51, 52]. In contrast, endurance-trained athletes have a greater capacity to oxidize lipids during a hyperinsulinemic/euglycemic challenge as compared to sedentary and obese individuals [53]. This observation coincides with others that exercise training enhances lipid oxidation at rest and during low intensity exercise [54, 55]. Training increases the reliance on lipid oxidation at the same workload due to various adaptations. An increase in maximal VO_2 and mitochondrial enzymes [56] as well as reduced hepatic gluconeogenesis, due to dampened

sympathetic nervous system activity [57], both contribute to increased lipid oxidation at low exercise intensities. Well-trained individuals also have a capacity to perform at higher absolute workloads, which is a direct effect of being able to rely on more carbohydrate oxidation during peak performance. The greater capacity to utilize carbohydrates during intense exercise observed in athletes is a consequence of greater intramuscular glycogen storage [58], and a shift in muscle fibers from IIb to IIa [59] and an increase in the proportion of type I fibers [60-62]. Lifelong endurance athletes also have more type I fibers than younger athletes [63]. Although muscle fibers are categorized based on contractile proteins, an increase in the ratio of type I to type IIb fibers is favorable for glucose metabolism since type I fibers have more insulin receptors [64] and glucose transporter type 4 (GLUT4) abundance [65]. Due to these adaptations to exercise, athletes exhibit enhanced metabolic flexibility while the consequence of metabolic disorders is metabolic inflexibility.

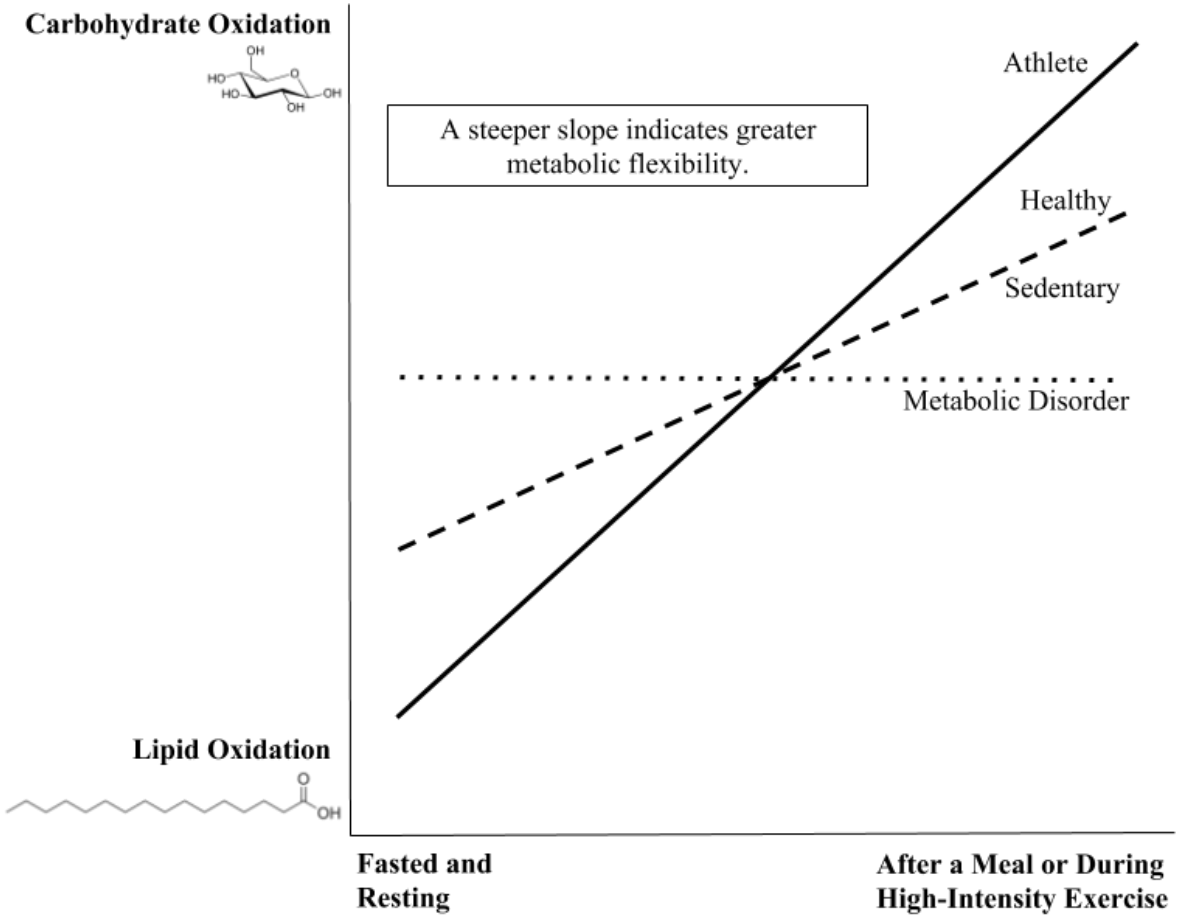

Figure 2:

Metabolic Flexibility is the Capacity to Alternate between Oxidizing Lipids or Carbohydrates According to the Supply and Demand of Energy.

Figure heavily inspired by previous depictions [48, 66].

Nutritional status modulates the improvements to metabolic flexibility induced by training. A bout of exercise leads to greater transcriptional activation of genes involved in the adaptive response if muscle glycogen is low [67-71]. Unfortunately, low muscle glycogen also blunts the capacity to perform high intensity exercise [72, 73]. During exhaustive aerobic activity, VO_2 remains stable although RQ falls as muscle glycogen is depleted until exercise cannot be continued [74]. In order to maximize muscle glycogen, and thereby high-intensity exercise performance, endurance athletes have followed various forms of high-carbohydrate diets in the days leading up to a competition [58, 74-76]. Alternatively, athletes can prolong high-intensity activity if they consume carbohydrates during the exercise [77]. For optimal post-exercise recovery, the International Society of Sports Nutrition recommends carbohydrate and protein intake in order to maximize glycogen repletion, minimize muscle damage, and maximize muscle-protein synthesis in the case of resistance exercise [78]. Although performance suffers if one performs exercise while in a fasted and glycogen-depleted state, doing so heightens the adaptations that lead to enhanced metabolic flexibility.

In addition to the changes described above, exercise may alter the epigenetic landscape of skeletal muscle. Epigenetic modifications influence gene function without altering the DNA sequence and are preserved in daughter cells after mitosis or meiosis. DNA methylation is one such modification that alters gene transcription and is the result of cytosine methylation by DNA methyltransferases. Germane to this thesis is the finding that exercise modulates DNA methylation [79] with subsequent changes in gene expression [80]. In contrasting research, 12 weeks of exercise training in young and old individuals resulted in only small changes in DNA methylation that did not correlate to changes in gene transcription [81]. In another multi-omics study, exercise did induce changes to DNA methylation in some genes as well as RNA expression of other genes, but the two phenomena did not affect the same genes [82]. The most compelling research to date linking exercise to hypomethylation of DNA with subsequent increases in mRNA expression gathered samples at several timepoints and linked the changes in DNA methylation to exercise intensity [80]. Research by several of the same investigators (some of whom are colleagues with the author of this thesis) identified that a high-fat diet alters the capacity for exercise to alter DNA methylation [83]. Other research studies have found that exercise induces shifts in DNA methylation measured from blood samples [84-86]. Changes to DNA methylation in whole blood may be due to altered cell population in circulation, rather than changes within individual cells, and may not reflect the DNA methylation status of skeletal muscle. There is a lack of consensus regarding how exercise modulates DNA methylation in skeletal muscle, and how DNA methylation changes affect metabolic flexibility.

In addition to exercise, caloric restriction enhances the metabolic health and lifespan of various non-human organisms, presumably by altering metabolic flexibility. Caloric restriction may exert some of its benefits through epigenetic mechanisms, although there is a deficit of human research to address this issue [87]. Nonetheless, diet-induced weight loss associates with alterations in DNA methylation measured in blood [88]. How fasting and exercise interact to alter DNA methylation is unsettled.

In light of the research implicating exercise and caloric restriction as modulators of DNA methylation and metabolic health, superimposing fasting and exercise bouts may lead to augmented adaptations to exercise. Due to the energetic stress induced by superimposing exercise and fasting, AMPK signaling may also be upregulated with effects on metabolic flexibility. The second and third papers of this thesis explore the role of AMPK in regulating skeletal muscle metabolism with an emphasis on identifying new AMPK targets.

1.3 AMPK: A PROTEIN COMPLEX AT THE CENTER OF ENERGY BALANCE

ATP hydrolase (ATPase) converts adenosine triphosphate (ATP) to adenosine diphosphate (ADP) in an exothermic reaction. If ATP levels are lower than ADP levels, adenylate kinase catalyzes the conversion of two ADP molecules to form a single ATP molecule and one molecule of adenosine monophosphate (AMP). The breakdown of ATP to ADP or AMP releases sufficient energy to drive various cellular processes, and specifically two important steps in excitation-contraction coupling: the segregation of Ca^{2+} ions into the sarcoplasmic reticulum via Ca^{2+} ATPase activity, and the release of myosin from actin filaments by myosin ATPase. The high production of AMP during exercise, coupled with the falling levels of ATP, leads to activation of AMPK by AMP in skeletal muscle [89]. Additionally, when energy levels in the cell are low, liver kinase B1 phosphorylates and activates AMPK [90, 91]. Calcium flux intracellularly during contraction activates calcium/calmodulin-dependent protein kinase kinase 2, which can also phosphorylate AMPK [92]. Because glycogen binds AMPK, as exercise persists and intracellular glycogen levels become depleted, AMPK molecules are unbound and available for activation [93]. The activation of AMPK in skeletal muscle during exercise is adaptive for organisms, since AMPK activity leads to a shift in balance of intracellular metabolism away from ATP-utilizing processes toward ATP-generating processes.

AMPK activation leads to a net increase in lipid oxidation, a cellular process that generates 129 molecules of ATP per molecule of palmitic acid catabolized. AMPK phosphorylates acetyl-coenzyme A carboxylase (ACC), inactivating the enzyme, and thereby reducing the ATP-depleting process of lipogenesis by inhibiting the formation of malonyl-coenzyme A (MCoA) [94]. ACC inactivation by AMPK also increases lipid oxidation, since MCoA allosterically inhibits carnitine-palmitoyl transferase 1 (CPT1) from importing fatty acids into the mitochondria [95]. AMPK activation also activates autophagy by phosphorylating Unc-51 like autophagy activating kinase, leading to increases in energy levels by recycling lipid and protein from cellular components [96].

In addition to its effects on lipid metabolism, AMPK activation enhances glucose uptake. Acutely, treatment of human skeletal muscle with an AMPK activator, aminoimidazole-4-carboxamide ribotide (AICAR) enhances glucose uptake [97]. Glucose uptake is a function of cell-surface GLUT4, and the intracellular GLUT4-containing vesicles are mobilized to the membrane after inactivation of Rab GTPase-activating proteins including TBC1 domain family member 1 (TBC1D1) and TBC1D4 [98, 99]. Exercise-induced AMPK activity correlates with inactivating phosphorylation of TBC1D1 and TBC1D4 in human skeletal

muscle [100], which indicates that AMPK-dependent phosphorylation of the TBC1 family enzymes mediates the increases in glucose uptake induced by AICAR treatment.

AMPK has important regulatory functions in non-skeletal muscle tissue as well. As little as 24–48 hours of constitutively active AMPK in rodent liver leads to hypoglycemia and fatty liver [101]. These effects are in contrast to what occurs in skeletal muscle, where continuous AMPK activity results in increased glycogen storage, an enhanced capacity to oxidize lipids, and a greater reliance on lipid oxidation to meet the demands of exercise [102]. An important difference between these tissues is that AMPK is activated during fasting in liver [103], but not in skeletal muscle [104]. Furthermore, fasting does increase AMPK in the hypothalamus, and hypothalamic expression of constitutively active AMPK leads to increased food intake and bodyweight [105]. The differential regulation and effects of AMPK across tissues is physiologically adaptive; a local low-energy signal induces the brain to increase calorie consumption, the liver to release glucose and ketones to the circulation, and the skeletal muscle to increase glucose uptake. Thus, AMPK activation in skeletal muscle acutely or chronically may enhance whole-body metabolic flexibility, whereas constant stimulation of AMPK signaling in other tissues may perturb metabolism.

Table 1:

Differential Activation of AMPK According to Stimulus and Tissue

Stimulus	**Fasting**			**Contraction**
Tissue	**Hypothalamus**	**Liver**		**Skeletal Muscle**
AMPK Activity	↑	↑	↔	↑
Effect	↑ caloric intake	↓ glycogen synthesis ↓ glycolysis ↑ ketogenesis	↔	↑ glucose uptake ↓ fatty-acid synthesis
Acute Result	↑ blood glucose	↑ blood glucose ↑ blood ketones	↔	↑ exercise prolongation
Chronic Result	↑ body weight	↓ liver glycogen ↓ blood glucose ↑ hepatic triglycerides	↔	↑ glycogen storage ↑ lipid oxidation

In skeletal muscle, the long-term effects of AMPK activation on metabolism are due to changes in transcriptional activity. AMPK activation leads to an increase in *GLUT4* gene expression, due to alterations in myocyte enhancer factor-2 binding to DNA [106]. Expression of the gene for peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1α) is also enhanced by AMPK activity [107], which leads to an increased abundance of mitochondrial proteins [108]. Elevated lipid oxidation subsequent to AMPK activation may also cause increases in nicotinamide adenine dinucleotide (NAD^+) which is necessary for sirtuin 1 (SIRT1)-mediated activation of PGC-1α [109].

The hypothesis that AMPK activation may increase SIRT1-mediated mitochondrial biogenesis is enthralling since mice with overexpression of SIRT1 are protected from developing metabolic dysfunction [110, 111]. Mitochondrial function, *per se*, is associated with walking performance in older adults [112, 113], and inversely associated with fatigability [114]. Although bodyweight tends to increase over the lifetime, mitochondrial respiration is more predictive of body mass index (BMI) than is chronological age [115]. Caloric restriction also activates SIRT1 [116], and humans who practice caloric restriction have elevated levels of SIRT1 and mitochondrial content in skeletal muscle [117]. In contrast, training over 16 weeks —but not caloric restriction— enhances mitochondrial content and activity in skeletal muscle, though both interventions improve insulin sensitivity [118]. AMPK activity may modulate the beneficial effects of caloric restriction since mice with skeletal muscle-specific genetic deficiency of AMPK subjected to caloric restriction actually develop impaired glucose tolerance [119].

AMPK is a particularly compelling protein to target for pharmacological intervention since its activation leads to glucose clearance from the blood, reduced intracellular lipogenesis, and increased mitochondrial biogenesis. Patients with T2D exhibit skeletal muscle insulin resistance [120]. Importantly, T2D patients retain the ability to increase AMPK-mediated glucose uptake [121], and the phosphorylation profile of AMPK and AMPK targets are similar between T2D patients and weight-matched controls after exercise or insulin stimulation [122]. Medications used to help T2D patients manage their disease also activate AMPK including the biguanidines [123, 124] and thiazolidinediones [125]. Even salicylate (aspirin) activates AMPK, at least in the liver [126].

The capacity for AMPK to induce glucose uptake in an insulin-independent manner deserves appreciation since it represents a point of overlap between otherwise opposing catabolic and anabolic processes. AMPK activity occurs within cells because of rapid energy depletion. In contrast, insulin stimulation occurs physiologically when there is a temporary surplus of energy-containing substrates. That both insulin stimulation and AMPK activation increase glucose uptake —despite originating from opposite extremes of the energy-availability spectrum— would be ingenious, if not for evolution.

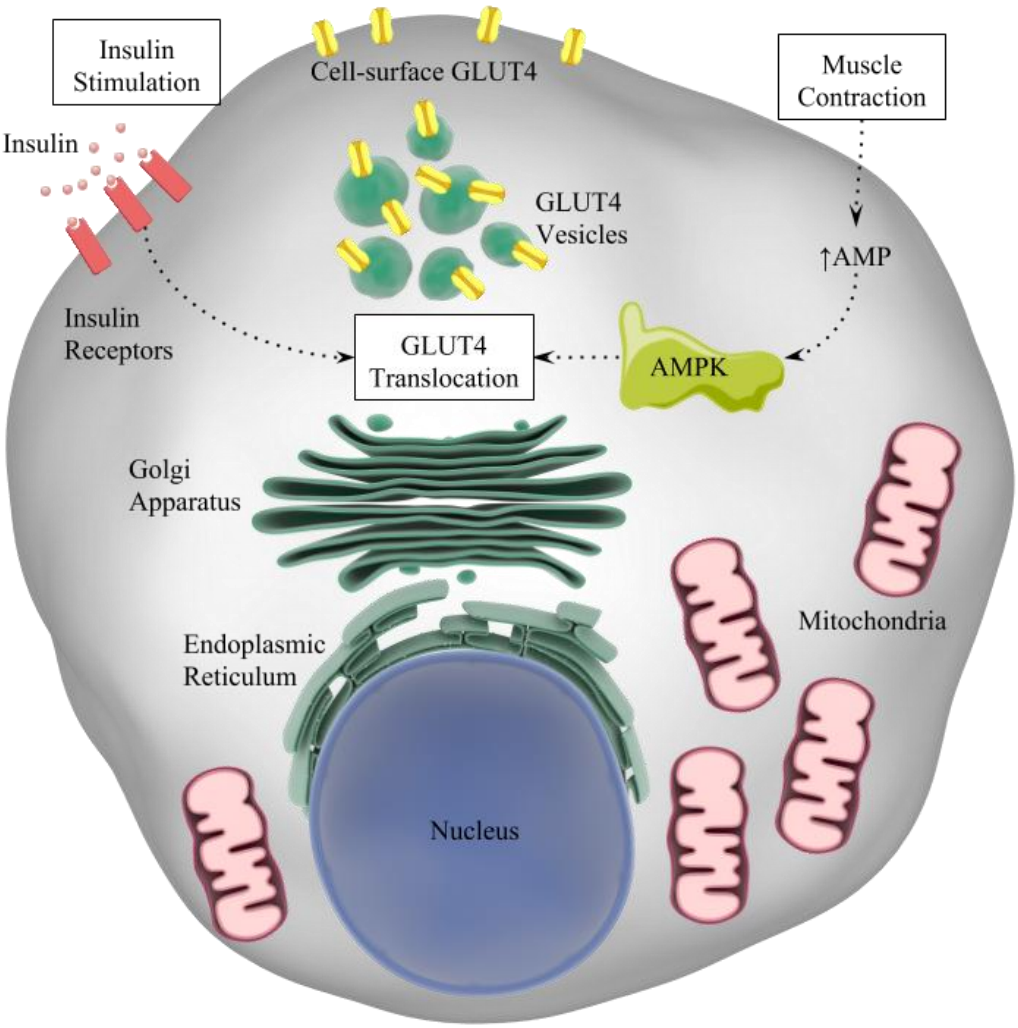

Figure 3:

AMPK Activation Induces GLUT4 Translocation Even in the Insulin-Resistant State.

1.4 AIMS OF THE THESIS

The central theory of this thesis is that exercise drives beneficial metabolic changes in skeletal muscle, largely due to AMPK activation. The hypotheses tested were:

1. Compared to exercising in a glycogen-repleted state, exercising in a glycogen-depleted state leads to greater AMPK activation, as well as reduces DNA methylation and enhances transcription of genes regulating lipid oxidation.
2. In skeletal muscle, AMPK activation and insulin stimulation antagonistically regulate focal adhesion kinase (FAK), a protein regulating cell growth and motility.
3. AMPK activity inhibits the expression of the gene coding for ganglioside-induced differentiation-associated protein 1 (*GDAP1*) in skeletal muscle, and this gene plays a role in maintaining mitochondrial function.

2 METHODS

2.1 HUMAN SUBJECTS

This thesis reports data from three groups of men who donated skeletal muscle tissue after giving informed consent. In the first study, seven Australian endurance athletes participated in an exercise and diet protocol. In the second paper, 11 healthy Swedish men donated skeletal muscle biopsies for use in research using *ex vivo* stimulation. In the final paper, archived samples from subjects that in previous research were analyzed [127]; specifically, 12 T2D patients and 12 controls with normal glucose tolerance donated biopsies at rest, immediately after exercise, and three hours later.

Table 2:

Participant Characteristics for Papers 1 and 2

	Paper 1	**Paper 2**
Age (years)	29 ± 5	50.6 ± 2.4
Body mass (kg)	76.9 ± 9.1	81.4 ± 3.3
Height (cm)	Not measured	179.9 ± 2.4
BMI (kg/m^2)	Not measured	25.1 ± 0.6
Waist-to-hip-ratio	Not measured	0.89 ± 0.01
VO_{2Peak} (mL/kg/min)	6.07 ± 4.0	Not measured
Peak power output (W)	422 ± 39	Not measured
Systolic blood pressure (mmHg)	Not measured	125.0 ± 3.9
Diastolic blood pressure (mmHg)	Not measured	79.5 ± 1.8
Fasting plasma glucose (mmol/L)	Not measured	5.3 ± 0.1
Fasting insulin (pmol/L)	Not measured	49.9 ± 7.9
HbA1C (%)	Not measured	5.3 ± 0.1
HbA1C (mmol/mol)	Not measured	34.5 ± 0.9
HDL-C (mmol/L)	Not measured	1.3 ± 0.1
LDL-C (mmol/L)	Not measured	3.9 ± 0.1
Total cholesterol (mmol/L)	Not measured	5.7 ± 0.1
Triacylglecerol (mmol/L)	Not measured	0.9 ± 0.2

All values reported as mean ± standard deviation.

Table 3:

Participant Characteristics for Paper 3

	Normal Glucose Tolerance	Type 2 Diabetes
Age (years)	59 ± 2	58 ± 2
Body mass (kg)	84.8 ± 3.4	86.4 ± 4.1
Height (cm)	179 ± 2	177 ± 2
BMI (kg/m^2)	26.4 ± 1.0	27.6 ± 1.0
Body fat (%)	27 ± 2	30 ± 2
VO_{2Peak} (mL/kg/min)	39.3 ± 2.35	32.2 ± 2.83
Peak power output (W)	253 ± 14	188 ± 16*
Peak heart rate (beats/min)	169 ± 3	166 ± 5
Systolic blood pressure (mmHg)	127 ± 4	131 ± 4
Diastolic blood pressure (mmHg)	79 ± 2	81 ± 3
Fasting plasma glucose (mmol/L)	5.0 ± 2.2	7.7 ± 0.5*
2 hour plasma glucose (mmol/L)	5.6 ± 0.5	14.3 ± 1.1*
Fasting insulin (pmol/L)	42.4 ± 6.3	68.8 ± 7.6*
HOMA-IR	1.38 ± 0.24	3.30 ± 0.53*
HbA1C (%)	5.3 ± 0.1	6.6 ± 0.3*
HbA1C (mmol/mol)	35 ± 1	49 ± 3*
HDL-C (mmol/L)	1.39 ± 0.12	1.22 ± 0.08
LDL-C (mmol/L)	3.24 ± 0.18	2.15 ± 0.19*
Total cholesterol (mmol/L)	5.07 ± 0.21	3.94 ± 0.20*
Triacylglecerol (mmol/L)	0.96 ± 0.10	1.26 ± 0.16

All values reported as mean ± standard deviation.

* Indicates significant difference between groups by an unpaired t-test

2.2 EXERCISE AND DIET INTERVENTIONS

For the first paper of this thesis, participants visited the research facility for two overnight experimental trials in a randomized crossover design. In both trials, participants consumed standardized meals, engaged in an exercise bout, stayed overnight in the laboratory, and engaged in a second exercise bout on the second day. In one arm of the crossover design, participants did not receive calories between the exercise bouts. In the other arm of the study, participants ingested calories in between the exercise bouts before going to sleep on the first day of the visit. The amount of food given to participants was isocaloric and had the same macronutrient profile between the visits. The experimental manipulation was whether participants received all of their calories before the first exercise bout on the first day, or if the calories split and instead ingested before and after the first exercise bout. In both arms of the study, participants donated blood that was later analyzed for changes in metabolic substrates and hormones. Participants also donated muscle biopsies that were rapidly frozen, and then split into aliquots for subsequent analysis. Biopsies were analyzed for changes in protein abundance and signaling by Western blot analysis, as well as for changes in gene expression using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Other aliquots of the biopsies were used to interrogate changes in DNA methylation in promoter regions of key metabolic genes.

For the second paper of the thesis, participants reported to the laboratory after an overnight fast to donate muscle biopsies from *vastus lateralis*. The participants in that research did not engage in other diet or exercise interventions.

For the third paper of the thesis, gene expression of *GDAP1* was measured in the *vastus lateralis* from T2D patients or individuals with normal glucose tolerance before and after an exercise bout. Other data from these participants has previously been reported [127]. Participants in the study donated a *vastus lateralis* muscle biopsy after an overnight fast (and abstention from metformin in the T2D group). The participants returned to the laboratory at least five days later and donated two additional muscle biopsies, immediately after 30 minutes of exercise and three hours later. The biopsies were subsequently processed for analysis of gene expression by RT-qPCR.

2.3 DNA METHYLATION

In the first paper of the thesis, the DNA methylation status of genes regulating metabolic function was interrogated. A literature search identified candidate genes and specific promoter regions for examination. A commercially available kit extracted DNA from the muscle biopsies. An enzymatic reaction bisulfite-converted the DNA, leading to conversion of unmethylated cytosines to uracils.

A polymerase chain reaction (PCR) with custom-designed oligonucleotides amplified the regions of interest identified from the literature search. The custom oligonucleotide targeting the reverse strand of bisulfite-converted DNA contained a biotin tag at the 5’ end, which was necessary for successful execution of downstream pyrosequencing.

Visual inspection of an aliquot of amplified DNA on an agarose gel cast with a fluorescent nucleic acid dye exposed to ultraviolet light verified amplification and specificity of the PCR. The amplified DNA was pyrosequenced and the proportion of methylated cytosines in the genomic DNA was determined by calculating the ratio of cytosines to tyrosines at each variable locus.

2.4 RT-QPCR

All three papers of this thesis utilize gene expression as a readout. Commercially available kits extracted RNA into water. Concentrations of RNA in different samples were determined via spectrophotometry. Reverse transcription (RT) was used to generate cDNA by loading reaction tubes with equal concentrations of RNA for samples from the same experiment. After cDNA synthesis, a portion of each sample was pooled so as to make a sample which would be used to construct a standard curve for the quantitative PCR (qPCR) step to come. A serial dilution of the pooled sample was used to make the points of the standard curve. All other samples were diluted 20-fold so as to minimize the effects of RT reagents in the subsequent qPCR. Custom-designed oligonucleotides were mixed with the samples and SYBR green reagents (Thermo Fisher Scientific, Waltham, MA) drove the qPCR. Specificity of oligonucleotides was verified by conducting a melt-curve analysis after the qPCR. In addition to candidate genes, reference genes were also analyzed in a separate qPCR. All data reported in the papers have been normalized to the abundance of reference genes.

2.5 WESTERN BLOT ANALYSIS

To investigate alterations in protein abundance and signaling, Western blot analysis was used in all three papers. Skeletal muscle biopsies from humans, whole muscle from mice, or primary human skeletal muscle cells were homogenized in a buffer containing protease inhibitors. Samples were centrifuged and supernatants separated from the debris pellet formed during centrifugation. A portion of the supernatant was used for quantifying protein concentration using a colorimetric Bradford assay or bicinchoninic acid assay.

Samples were diluted in reducing Laemmli buffer to equal concentrations and heated to 57°C for 20 minutes or longer. Equal volumes of samples were loaded into precast gels and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed. Proteins were transferred from gels to polyvinylidene difluoride (PVDF) membranes by electroblotting. PVDF membranes were reversibly stained with Ponceau S stain to assess equal loading of lanes with samples qualitatively. The Ponceau S stain was removed by raising the pH using repeated washes with pH 7.6 tris-buffered saline with Tween-20 (TBST).

To prevent non-specific binding of antibodies in subsequent steps, membranes were blocked in 7.5% non-fat dry milk in TBST. The membranes were exposed to primary antibodies targeting specific proteins of interest overnight at 4°C. The following day, membranes were washed several times in TBST and then exposed to horseradish-peroxidase (HRP) conjugated secondary antibodies specific to the primary antibodies used in the previous overnight incubation. The secondary antibody treatment was conducted at room temperature for at least

one hour. Membranes were washed several times in TBST and then treated with enhanced chemiluminescence (ECL) substrate. Because the HRP and ECL chemically reacted to generate light, the membranes were then transferred to a darkroom and exposed to x-ray film.

The x-ray film was developed in the darkroom and subsequently scanned on a flatbed scanner suitable for image acquisition to permit downstream densitometry. The scanned images were used to quantify optical density of bands on the x-ray films according to which sample to which they corresponded. Molecular ladders were used when loading the polyacrylamide gels and marked on the x-ray films for orientation. Images that were overexposed were not used for quantification.

2.6 *EX VIVO* STIMULATION OF HUMAN SKELETAL MUSCLE BIOPSIES

In the second paper of the thesis, a previously developed open-muscle biopsy technique [128-130] was used to study the influence of insulin- and AICAR-stimulation on FAK phosphorylation in human skeletal muscle. The technique was undertaken under aseptic conditions. After local delivery of anesthetics to *vastus lateralis* muscle of volunteers, a surgeon made an incision through the skin and fascia to reveal the skeletal muscle tissue. The skeletal muscle was clamped at resting length, and muscle fibers were dissected out and transferred to oxygenetaed Krebs-Henseleit bicarbonate buffer that was supplemented with mannitol and glucose.

The fibers were transported from the hospital to the research facility where they were then trimmed of connective tissue and mounted on Plexiglass clips as strips approximating one centimeter in length. The muscle strips had a final weight of ~20 milligrams. Muscle strips were incubated for 30 minutes in a recovery buffer, 20 minutes in a treatment buffer containing insulin or AICAR, 10 minutes in a glucose-free rinse buffer, and finally 20 minutes in a buffer containing ^{14}C-labeled mannitol and ^{3}H-labeled 3-*O*-methylglucose. The muscle strips were subsequently frozen in liquid-nitrogen cooled clamps and saved for later analysis.

The gene expression of these strips was not explored in this thesis, but has been explored in another publication [131]. In this thesis, protein signaling and glucose transport were assessed by Western blot analysis and scintillation counting of radioactive isotopes, respectively. Specifically, scintillation counting of ^{3}H and ^{14}C revealed how much 3-*O*-methylglucose and mannitol was taken into the fibers, respectively. Because mannitol cannot be taken up by intact muscle cells, the difference in the concentrations calculated from the scintillation counts was used to infer how much 3-*O*-methylglucose was taken up by the cells.

2.7 GENETICALLY MODIFIED MICE

To assess how AMPK activity influences *GDAP1* expression in skeletal muscle, two different strains of genetically modified mice previously developed were used for the third paper of the thesis [132]. Both strains of mice have altered functionality of AMPK due to genetic manipulations of the γ3 subunit, which is predominantly expressed in skeletal muscle. One strain of mice has a deletion in the coding region for AMPKγ3, resulting in reduced AMPK

activity in skeletal muscle. The other strain has a transgene with a mutation causing an arginine to a glutamine switch at amino acid 225 in the translated polypeptide, resulting in an AMPK protein that is constitutively active due to an inability to take an inactive conformation. The strains are referred to as AMPK$\gamma3^{-/-}$ and AMPK$\gamma3^{R225Q}$, respectively.

In the third paper of the thesis, mice with these genetic modifications, and their respective wild-type littermates, were given free access to food or subjected to an overnight 12-hour fast. Following the overnight period, blood glucose was sampled from the tail, mice were anesthetized, and *gastrocnemius* tissues were harvested. Gene expression was measured by RT-qPCR as described earlier in this thesis.

2.8 PRIMARY HUMAN SKELETAL MUSCLE CELL CULTURE

The use of primary human skeletal muscle cells was central to the final two papers of the thesis. Primary cells used in the second paper were isolated from skeletal muscle tissue unused in the glucose transport assay described earlier. The primary cells used in the third paper were prepared and described by the research previously [133]. The satellite-cell isolation technique has been described [134]; biopsies were digested with collagenase and trypsin, supernatant containing satellite cells and fibroblasts were harvested and added to a petri dish for one hour to select against adherent fibroblasts, and the supernatant was finally collected from the dish to retrieve the satellite cells.

When primary human skeletal muscle cells were used in the thesis, satellite cells were grown and differentiated into myotubes according to methods described elsewhere [131]. Briefly, cells were seeded in uncoated plates or dishes and incubated at 37°C in the presence of a growth medium containing 20% fetal bovine serum (FBS). Once cells reached ~80% confluence, differentiation was induced by switching to a medium containing 2% FBS, 100 µg/mL apotransferrin, and a high dose of insulin (1.7 µM). Cells were incubated in this differentiation media for ~96 hours prior to being switched to an insulin-sensitizing media that was insulin-free. Experiments were carried out after ~96 hours in the final media. In all steps of cell growth and differentiation, cells received fresh media every 2–3 days.

2.9 GENE SILENCING IN CELLS

Manipulating gene expression in primary skeletal muscle cells was a central experimental technique used in the second and third papers of the thesis. Lipofection was utilized to deliver siRNA to cells in order to silence genes of interest as described elsewhere [135]. Specifically, cells were transfected on the first day of incubation with the low-insulin post-differentiation media, and again ~48 hours later. Final experiments were carried out ~48 hours after the second transfection. In all transfection experiments, cells were transfected with either siRNA directed against the gene of interest or a negative control siRNA.

2.10 METABOLIC PHENOTYPING OF CELLS

After silencing genes of interest, the metabolic phenotype of the primary human skeletal muscle cells was investigated in the final two papers of the thesis. Assays for palmitate oxidation and

glycogen synthesis were performed for both papers. For the final paper, assays assessing glucose oxidation, glucose uptake, lactate production, and mitochondrial function were also performed.

To assess palmitate oxidation, cells were incubated with ^{3}H-labeled palmitic acid in the presence of AICAR or differing doses of glucose. As the cells oxidized the palmitate, the ^{3}H was incorporated into water and released from the cells. Supernatant from the cells was harvested and separated from the palmitate with activated charcoal. The palmitate-free supernatant was then subjected to scintillation counting to assess palmitate oxidation.

Glycogen synthesis was assessed by exposing serum-starved cells to ^{14}C-labeled glucose in the presence of different doses of insulin. Cells were lysed, glycogen was precipitated and finally subjected to scintillation counting.

Like the glycogen synthesis assay, ^{14}C-labeled glucose was the tracer used to assess glucose oxidation. As the cells oxidized glucose, they released radiolabeled-CO_2 into the media. To capture the released CO_2, a small plastic cup was placed gently on top of the cells and cell plates were sealed with airtight plate sealers. After four hours of glucose oxidation, a hypodermic needle was used to deliver NaOH through the plate seals and into the plastic cup. Immediately after, HCl was delivered to the cell media, causing a drop in pH and consequently a release of CO_2 into the air above the media. By the law of mass action, the CO_2 in the air accumulated into the NaOH. After one hour, the plates were opened and the small plastic cups were transferred to scintillation vials for quantification of radioactivity.

The glucose uptake assay was conducted as previously described [135]. Cells were incubated for 1 hour in a glucose- and serum-free media prior to a 15-minute exposure to 2-[1,2-^{3}H]deoxy-D-glucose. This glucose analog can be taken up by the cells and phosphorylated by hexokinase, but is not further metabolized. Cells were rinsed with ice-cold phosphate-buffered saline (PBS), and lysed. Lysed cells were used in scintillation counting to determine glucose uptake.

The media from the one-hour incubation of cells in the glucose- and serum-free media in the glucose uptake assay was collected to assess lactate production. A colorimetric assay was used wherein lactate dehydrogenase converted lactate to pyruvate and NAD^{+}. The NAD^{+} could be detected by spectrophotometry and used to infer lactate production by the cells [136].

Mitochondrial function of cells was assessed by subjecting them to a stress test and measuring oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) by using a Seahorse analyzer (Santa Clara, CA). The analyzer measures OCR and ECAR via probes that detect changes in O_2 tension and pH in each well of a 24-well cell-culture plate. Additionally, the analyzer has several injection ports that allow for the timed delivery of different compounds to the cells in order to study how the cells respond due to various treatments. In the mitochondrial stress test, OCR and ECAR were measured at three timepoints prior to any pharmacological stimulation in order to establish baseline readings. Oligomycin, an ATP

synthase inhibitor, was then injected. After three more readings, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone was injected to uncouple proton flow from ATP synthesis in mitochondria. Three more readings were taken before an injection of rotenone and antimycin A, which block the electron transfer of mitochondrial complexes I and III to ubiquinone, respectively. A final three readings were made of OCR and ECAR before terminating the experiments. The alterations in OCR and ECAR under different conditions permitted the calculation of multiple parameters of mitochondrial function and oxidative metabolism in the cells.

All of the metabolic assays conducted in the cells were normalized to protein content as measured by a colorimetric Bradford assay or bicinchoninic acid assay.

2.11 CONFOCAL MICROSCOPY

Confocal microscopy was used to examine the effects of silencing *GDAP1* expression on mitochondrial structure in primary skeletal muscle cells. Approximately 48 hours after the final transfection of siRNA, cytoplasm, nuclei, and mitochondria were stained using protocols recommended by the dyes' manufacturers. After staining, cells were fixed by treating with 3.7% formaldehyde for 20 minutes and then washed several times with PBS.

Blue-fluorescing cell nuclei were located through the eyepiece of a confocal microscope using a 40X-magnification objective while exciting the Hoechst dye fluorophores using light at 361 nm λ. Next, simultaneous excitation of the nuclear dye and the mitochondrial dye was accomplished by stimulating with light at two wavelengths (361 nm λ and 644 nm λ). Software translated the emitted light (486 nm λ and 665 nm λ, respectively) into blue and white on a computer monitor, and focus was manually adjusted. A three-by-three tiling array of images was made by the software in order to get an overview of the cell population.

From the overview picture, at least four fused myotubes were selected for obtaining more-detailed images. The software moved the confocal microscope to the x,y-coordinates of the selected myotubes and focus in the z-axis was adjusted manually. Individual images of nuclei, cytoplasm, and mitochondria were obtained from each of the saved x,y,z-coordinates by sequentially exciting fluorophores with respective light sources and capturing emitted light in the appropriate range. A final overlay image was made by superimposing the three separate images obtained.

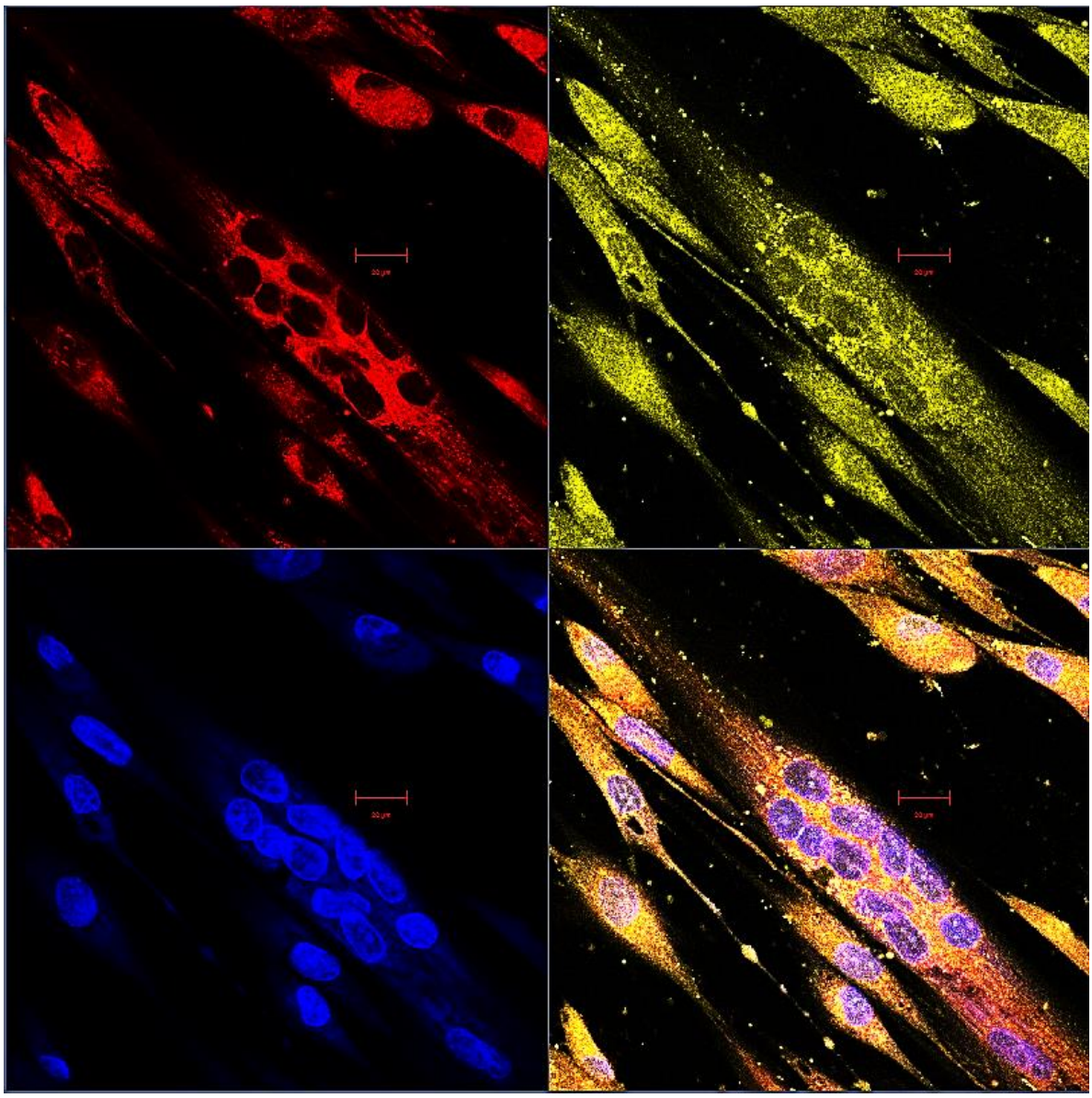

Figure 4:

Confocal Microscopy of Primary Human Skeletal Muscle Cells

Clockwise from bottom left: Nuclei, Mitochondria, Cytoplasm, and Overlay.

Scale bar is 20 µm.

To analyze the data, only the nuclei and mitochondrial networks were superimposed in a selection of images. These images were coded to mask the treatment associated with each image. Images were sent to collaborators who were assigned the task of determining if there were consistent qualitative differences between treatment groups in terms of mitochondrial network morphology. If there was consensus among the blinded researchers regarding mitochondrial network morphology according to experimental conditions, quantitative analysis would have been warranted and a non-biased method of obtaining images and quantifying mitochondrial structures would have been developed.

2.12 BIOINFORMATIC ANALYSIS AND USE OF PUBLIC DATA

The primary hypothesis for the third paper of the thesis was that overlaying data from publically available microarray studies would successfully identify genes regulated by AMPK activity. Data from three experiments reported in two publications were used to generate a list of candidate genes [137, 138]. The microarrays used to identify candidate genes were generated by experimentally manipulating AMPK activity in mouse skeletal muscle by injections with AICAR [137] or through genetic modifications to AMPK [138], previously described in this thesis [132]. To identify the candidate genes, expression analysis from the three datasets was normalized by the robust multi-array averaging (RMA) method [139]. The RMA method enables comparisons of gene expression between microarray studies by transforming the reads across all datasets to fit on the same scale. Candidate genes thought to be increased by AMPK activity were identified if they were significantly increased in all models of increased AMPK activation and significantly decreased in the model of AMPK inhibition. In contrast, candidate genes thought to be negatively regulated by AMPK activity were identified if they were significantly decreased in all models of AMPK activation and significantly increased in the model of AMPK inhibition.

The gene expression omnibus (GEO) and genome-wide association studies (GWAS) catalog were two other publically available tools used to investigate the role *GDAP1* plays in metabolic disorder [140, 141]. The GEO helped to identify research studies where *GDAP1* expression was reduced in mouse skeletal muscle due to acute fasting and lifetime caloric restriction [142, 143]. The GWAS catalog identified a dozen publications linking single-nucleotide polymorphisms in the *GDAP1* gene to signs or symptoms of metabolic disorder including waist circumference, blood pressure, and circulating fatty acids [144-155].

2.13 DATA ANALYSIS AND STATISTICS

In the second and third papers of the thesis, all inferential statistics were conducted using R, an open-source free-to-use computer programming language [156]. Several add-on libraries also facilitated data processing and analysis: “openxlsx” for reading and writing files to Microsoft Excel [157], “magrittr” and “tidyverse” for writing code in an easy-to-read manner and visualizing data [158, 159], and “ez” and “lawstat” for conducting inferential statistics [160, 161]. In the first paper, inferential statistics were performed using other commercial software, but the data interpretations for the DNA-methylation results were crosschecked by using R. In all papers, α was set to 0.05.

The data analysis consisted of first identifying which kind of parametric omnibus test was most appropriate for the study design (e.g., a t-test in a simple design or a 2-way mixed-model analysis of variance (ANOVA) in a more complex study design). After identifying which omnibus test was most appropriate, the assumptions of the specific test were identified and tested. When the assumptions were violated, if adjustments could be made to the omnibus test to correct for violations, adjustments were made. If adjustments could not be made because of the violated assumptions, an alternative non-parametric omnibus test was employed instead.

For example, in the case of a two-way mixed-model ANOVA, there are seven assumptions to test for before interpreting p-value outputs of the statistical test [162]. Three of these assumptions are regularly passed over by researchers: 1) the assumption that the dependent variable is normally distributed for each group analyzed (e.g., each combination of the two factors in the two-way mixed-model ANOVA), 2) the assumption that there is homogeneity of variance for each combination of the groups (also known as homoscedasticity), and 3) the assumption that the variances of the differences between the related groups of the within-subjects factors for all groups of the between-subjects factor is equal (this is also known as sphericity). In this thesis, when the assumption of normality was to be tested, a Shapiro-Wilk test was used. To test for homogeneity of variance, Levene's test was used. A Mauchly's test was used for assessing if the assumption of sphericity was violated. In cases where the assumption of normality could not be reasonably kept (i.e., Shapiro-Wilk's test yielded $p < 0.05$), a non-parametric omnibus test was selected to analyze the data. If Levene's test was significant, a White adjustment was made. If a Mauchly's test was significant, a Greenhouse-Geisser correction was made if the test statistic $\varepsilon < 0.75$, and a Huynh-Feldt correction was made otherwise.

Pairwise *post-hoc* tests were conducted with equal consideration for violations of test assumptions. The risk of reporting false-positive results was reduced by using the Benjamini-Hochberg false-discovery rate correction when interpreting the p-values from multiple *post-hoc* tests.

3 RESULTS

3.1 PAPER 1: EFFECTS OF SLEEPING WITH REDUCED CARBOHYDRATE AVAILABILITY ON ACUTE TRAINING RESPONSES

The primary finding from the first paper was that exercising while in a fasted state the morning after a glycogen-depleting exercise bout enhances the capacity of skeletal muscle to oxidize fats by increasing mitochondrial protein abundance and expression of genes involved in lipid oxidation. Specifically, the fasting protocol superimposed onto the exercise bouts led to increases in AMPK activity —as evidenced by increased phospho-AMPK^{Thr172} and phospho-ACC^{Ser222}— as well as the abundance of CPT1, and adipose triglyceride lipase. The genes for pyruvate dehydrogenase kinase 4 and fatty-acid binding protein 3 (*FABP3*) were also increased due to fasting. DNA methylation on the promoter for the *FABP3* gene tended to be decreased under the fasting state. Peroxisome proliferator-activated receptor delta expression was not significantly impacted due to fasting, although there was significantly increased DNA methylation in the promoter at the final timepoint. Together, these results demonstrate that combining exercise and fasting augments the adaptive response to exercise.

3.2 PAPER 2: FAK TYROSINE PHOSPHORYLATION IS REGULATED BY AMPK AND CONTROLS METABOLISM IN HUMAN SKELETAL MUSCLE

The hypothesis for the second paper was that insulin- and AMPK-stimulation would regulate FAK activity in opposing manners in human skeletal muscle. Surprisingly, an increase in phospho-FAK^{Tyr397} due to insulin stimulation was not observed, although this effect occurs in other models. However, AICAR treatment did reduce phospho-FAK^{Tyr397} in human skeletal muscle biopsies treated *ex vivo*. AICAR and serum-starvation also lead to decreases in phospho-FAK^{Tyr397} that were inversely correlated with phospho-ACC^{Ser222}. Reducing FAK activity by silencing its gene, *PTK2*, increases lipid oxidation and decreases glycogen synthesis in primary human skeletal muscle cells. Silencing *PTK2* did not alter AICAR-stimulated changes in phospho-ACC^{Ser222} nor insulin-stimulated changes in phosphorylation of protein kinase B. These data reveal that, in human skeletal muscle, FAK is an AMPK-regulated protein that controls fat oxidation.

3.3 PAPER 3: LIPID OXIDATION IN SKELETAL MUSCLE IS IMPAIRED DUE TO *GDAP1* SILENCING, AN AMPK-REGULATED GENE

In the final paper, *GDAP1* was identified as an AMPK-regulated gene that plays a role in lipid oxidation. A bioinformatic analysis revealed *GDAP1* as a gene under the control of AMPK. Validation experiments showed that *GDAP1* expression in skeletal muscle inversely correlates to AMPK activity. Furthermore, silencing *GDAP1* in primary skeletal muscle cells reduces lipid oxidation as well as non-mitochondrial respiration. Finally, AMPK activation and silencing *GDAP1* independently alter the expression of circadian genes. Unlike in nerve cells, *GDAP1* expression in skeletal muscle cells does not appear to alter mitochondrial form or function. In summary, *GDAP1* is regulated by AMPK in skeletal muscle, plays a role in non-mitochondrial metabolism, and interacts with the circadian machinery.

4 DISCUSSION

It has been recognized for centuries, if not millennia, that exercise is essential for wellbeing. In the 1700s, the "father of orthopedics", Nicolas Andry de Boi-Regard, theorized that exercise enhances health [163]. Socrates, the ancient Greek philosopher, also recognized that maintaining physical fitness was key to maintaining health, happiness, and preparedness for life's struggles [164]. Even more-ancient non-Occidental traditions have emphasized physical activity as a matter of course, and this is echoed in today's manifestations of yoga and other reverent exercise practices. Among the beneficial effects of exercise is its capacity to profoundly improve the metabolic health of individuals.

The research conducted in the past decades has begun to reveal the mechanisms responsible for the metabolic improvements induced by exercise. Among the important discoveries has been that AMPK is activated due to rapid energy turnover in cells, and is a key driver for adapting to exercise. Additionally, because AMPK activation increases glucose uptake —even in insulin-resistant patients [121]— investigation of this protein is a central focus in metabolic research. However, the physiological and molecular processes involved in exercise adaptation and AMPK activation are still incompletely resolved.

The work in this thesis is directed toward revealing previously undescribed effects of exercise and AMPK activation in human skeletal muscle. First, a novel strategy to enhance exercise adaptations by fasting between training sessions is investigated. Second, a role for AMPK to inhibit the activity of FAK in skeletal muscle is described. Finally, *GDAP1* is identified as an AMPK-regulated gene that plays a role in non-mitochondrial lipid oxidation and the circadian rhythm of skeletal muscle cells. Each of the papers presented in this thesis further contribute to the field of metabolic research.

4.1 PAPER 1: PROS AND CONS OF FASTING AFTER TONIGHT'S EXERCISE TO ENHANCE THE EFFECTIVENESS OF TOMORROW'S WORKOUT

Markers of endurance training are acutely upregulated if carbohydrates are restricted for the three-hour timeframe after exercise [165], and the transcriptional response to exercise is intensified if skeletal muscle glycogen stores were previously depleted [68, 69, 71]. The results described from the first paper in the thesis are in agreement with this previous research. The earlier studies reported that repeated exercise bouts conducted in the same day enhance the adaptive response due to low skeletal muscle glycogen. The new results from this thesis reveal that individuals may split two workouts between an evening session and a morning session while still gaining the adaptive benefits, so long as calories are restricted between the exercise bouts. Fasting between the exercise bouts also leads to changes in DNA methylation in targeted genes, which may alter transcriptional activity.

A similar intervention paradigm was extended over a three-week period in follow-up research [166]. That research revealed that regular engagement in morning exercise in the glycogen-depleted state after an exercise bout the day before led to enhanced performance on measures of endurance as well as reduced fat mass [166]. In the follow-up research, it was reported

that sleep quality was not negatively impacted by sleeping with low glycogen stores over the three-week period [167]. However, it was not a crossover study, and the authors report data indicating that before the intervention began, the group of individuals in the low-glycogen arm of the study had greater sleep efficiency and more sleep time as compared to individuals in the control group. More importantly, the low-glycogen group did exhibit reduced sleep efficiency while engaging in the diet intervention. Abstention from carbohydrates after an evening exercise does enhance the adaptive response elicited from a training session conducted the following morning, but it may also impair sleep quality.

The benefits of late-day exercise and fasting must be carefully weighed against potential drawbacks. Longer periods of uninterrupted sleep are associated with reduced blood sugar levels [168]. Experimentally misaligning the circadian rhythm by shifting light exposure in humans by 12 hours impairs glucose tolerance and insulin sensitivity [169]. Additionally, variations in the melatonin receptor 1B gene associate with hyperglycemia in bipolar patients [170]. Given that perturbations to normal circadian function negatively affect glucose homeostasis, the fasting and exercise paradigm used to enhance skeletal muscle adaptations may be contraindicated if it leads to impaired sleep quality.

In light of these negative consequences on glucose homeostasis by disrupted sleep and circadian rhythm, alternatives to fasting after an evening exercise bout may be preferred to elicit the beneficial effects of training with low muscle-glycogen content. Prescribing caloric restriction by 25% over a two-year period led to reductions in body weight without negatively impacting aerobic fitness, despite actual caloric restriction only reaching ~10% [171]. Caloric restriction even enhances sleep quality over a two-year period [172]. Similar to exercise, some of the beneficial effect of caloric restriction is can be attributed to AMPK activation [119].

Another alternative to enhance metabolic fitness may be time-restricted feeding. Unfortunately, the research into the effects of time-restricted feeding on human physiology has been limited, and the studies that have been carried out have been non-experimental in nature. An exception has been a crossover study revealing that patients with T2D lose more weight, reduce blood glucose, and enhance insulin sensitivity if they consume a reduced-calorie diet as two meals in the first half of the day instead of as six meals spread throughout the day [173]. The benefits of resistance training are either unaffected [174] or enhanced by [175, 176] time-restricted feeding. Research in mice is also promising. Mice with free access to a high-fat diet develop impaired metabolic phenotypes, although restricting food access to only a few hours a day protects against this [177, 178]. Furthermore, entrainment of the circadian rhythm in mice is achieved via caloric restriction or time-restricted feeding alike [179]. Most mouse strains used in laboratory settings have no melatonin production or function [180]. Since melatonin is a well-conserved circadian hormone whose endogenous production is entrained by light exposure, translating circadian research using nocturnal rodents with nonoperational melatonin systems to diurnal humans should only be done with a great deal of caution.

Even if the diet and exercise intervention used in the first paper of this thesis is not the perfect means to enhance metabolic fitness or adaptations to exercise, the research was unique since it explored how DNA methylation changed in response to fasting between exercise bouts. Unfortunately, neither DNA methylation nor other epigenetic modifications were characterized in the follow-up research that took place over three weeks of training [166, 167]. There are only a handful of publications characterizing the DNA methylation response to exercise or diet interventions in human skeletal muscle, making this work relatively unique.

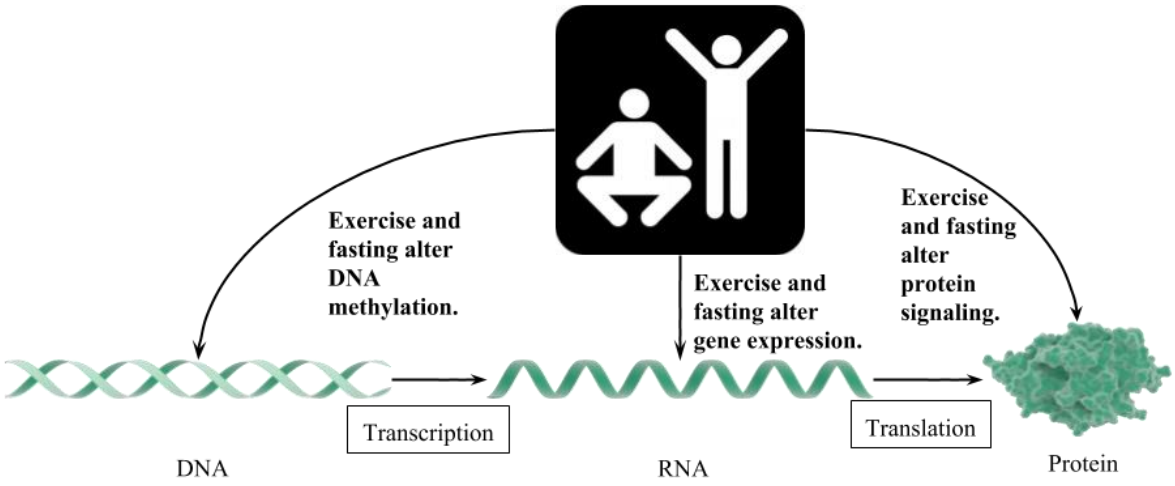

Figure 5:

Exercise and Fasting Impact Every Aspect of the "Central Dogma of Biology".

Research into epigenomic effects of lifestyle modifications in humans is limited due to two main factors: financial barriers to conducting the research and an inability to directly tie observations to a physiological readout. In the first paper of the thesis, DNA methylation of specific target-gene promoters was interrogated, which was not cost-prohibitive. However, using techniques to study the entire DNA methylome would have increased the cost of the research by at least an order of magnitude. Furthermore, any data retrieved from the epigenomic analysis would still have been difficult to tie to the other readouts of the research in a mechanistic fashion. Although DNA methylation in a promoter region may lead to inhibition of gene transcription, such data can only be correlational without having experimental methods to manipulate DNA methylation *in vivo*. Thus, although the research conducted in the first paper of the thesis provided novel insights into epigenetic effects in skeletal muscle due to fasting between exercise bouts, the shortage of other similar research makes the observations difficult to contextualize.

In contrast, there is a wealth of information in the scientific literature regarding how exercise influences AMPK functionality. The second paper of the thesis adds to this body of research by exposing a role for AMPK activity in the dephosphorylation and inhibition of FAK

signaling in human skeletal muscle. The final paper reveals a role for AMPK in inhibiting *GDAP1* gene expression, also in human skeletal muscle.

4.2 PAPER 2: FAK'S ROLE IN ALTERING SUBSTRATE UTILIZATION HAS IMPLICATIONS FOR METABOLIC DISORDER

Despite FAK being a known mediator of insulin signaling in rodent models and immortalized cells [181-189], the influence of insulin stimulation on FAK in human skeletal muscle had not been previously described. The second paper of this thesis addresses this gap in the literature by revealing that neither primary human skeletal muscle cells, nor insulin-sensitive human muscle tissue stimulated *ex vivo*, have alterations in FAK phosphorylation in response to insulin stimulation.

Furthermore, although FAK activation has been observed due to stretch and unloading-reloading protocols [190-192], a direct link between AMPK activity and FAK had not been previously described. The research in this paper shows that AMPK activation in human skeletal muscle reduces FAK phosphorylation and, thus, activation. This research was taken a step further and demonstrated that reducing FAK (by silencing its gene, *PTK2*) leads to increased lipid oxidation and reduced glycogen synthesis.

These findings regarding AMPK regulation of FAK, although from skeletal muscle, may have broader implications for cancer therapies. FAK activity is predominantly studied in proliferative and invasive cell types, and FAK inhibitors are a strain of pharmaceuticals that are at varying stages in clinical trials. Cancer cells are typified by prioritizing glucose oxidation over lipid oxidation, a phenomenon known as the Warburg effect, and this is hypothesized to be due to insufficient lipid utilization by mitochondria [193]. In this sense, cancer can be thought of as a complex condition with characteristics of a metabolic disorder, since cancer cells have altered metabolic flexibility. That FAK knockdown in skeletal muscle cells changes the metabolic preference of cells toward lipid oxidation is remarkable. Intriguingly, metformin, a drug prescribed to treat T2D patients, activates AMPK and has some use in combating cancer progression despite not accumulating in tumorous tissues in humans [194]. Furthermore, cancer cells treated with metformin have reduced FAK phosphorylation [195, 196].

The specific manner by which AMPK activity leads to dephosphorylation of FAK is unclear. Protein phosphatase 2 and dual-specificity phosphatase (DUSP) may be responsible since they can be activated by AMPK [197, 198]. DUSP specifically ought to be examined in future research, since FAK inhibitors increase the expression of *DUSP1* and *DUSP5* [199].

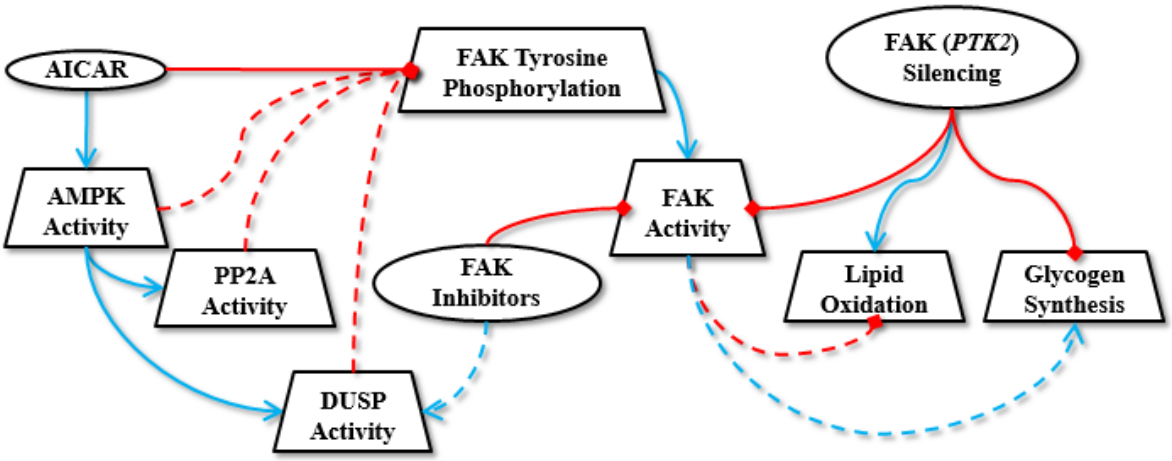

Figure 6:

AMPK-Mediated FAK Activity and Lipid Oxidation

Blue arrows indicate activating effects and red diamonds indicate inhibiting effects.

Solid lines indicate observed effects and dashed lines indicate plausible effects.

These studies indicate that further characterization of the complex interaction of AMPK with targets in various tissues will likely lead to discoveries that can inform future treatments for chronic diseases typified by metabolic disruption. To this end, the third paper was focused on identifying novel candidate genes regulated by AMPK in skeletal muscle.

4.3 PAPER 3: *GDAP1* ALTERS NON-MITOCHONDRIAL METABOLISM PRIMARY IN HUMAN SKELETAL MUSCLE

The bioinformatic analysis identified several candidate genes that may be regulated by AMPK. Preliminary experiments validated a role for AMPK in regulation of several of these genes. Future research may therefore take this list of genes as a starting point for further exploration. Among the candidate genes was *GDAP1*. Mitochondrial dysfunction as a result of mutations in the *GDAP1* gene cause a neuromuscular disorder known as Charcot-Marie Tooth syndrome [200]. Though the GDAP1 protein also plays a role in insulin signaling in fruit flies [201], its role in human skeletal muscle had not been described. Because of its role in both mitochondrial function and as a mediator of insulin signaling, *GDAP1* was selected for further investigation.

Table 4:

Impact of AMPK Activity on Gene Expression

Human Gene Name	Predicted Effect	Observed in Mouse Models (n = 6-8)		Observed in Primary Human Myotubes (n = 3)
		Fasting Effect	Genetic Effect	
AAMDC	↓	↓	↓	↓
ASPH	↓	↔	↑	↓
DPP8	↓	↓	↓	↓
FAM20C	↓	↓	↑	↔
GDAP1	↓	↓	↓	↓
GPX3	↑	↑	↑	↓
HHATL	↓	↓	↓	Not expressed
KANSL1L	↓	↓	↓	↓
LPIN1	↑	↔	↑	↑
PHLDA3	↑	↔	↑	↔
UGP2	↑	↓	↓	↓

Arrow directions indicate the direction of gene expression change predicted by the bioinformatic analysis or observed in the validation experiments.

Blue and red backgrounds indicate the data from validation experiments supported or were contrary to the bioinformatic analysis, respectively.

In skeletal muscle cells, mitochondrial function remains intact when *GDAP1* is silenced. This is in contrast to nervous tissue, where GDAP1 primarily acts to maintain mitochondrial function [200, 202, 203]. Silencing *GDAP1* in skeletal muscle cells does not alter glycogen synthesis and actually impairs lipid oxidation. The Seahorse metabolic flux analyzer revealed that the cells have unaltered mitochondrial oxidation, although non-mitochondrial respiration is decreased. Confocal microscopy did not identify differences in mitochondrial networks. Thus, the role of GDAP1 in skeletal muscle is to modulate metabolism without affecting mitochondrial morphology.

GDAP1 also induces peroxisomal fragmentation in immortalized fibroblasts [204]. Under conditions of metabolic stress, the peroxisomes oxidize lipids in cells [205, 206]. The results in the third paper of the thesis suggest a peroxisome-centered role for GDAP1 in human skeletal

muscle. In the patients with T2D, increased *GDAP1* may be elevated as an adaptive response to oxidize lipids, whereas the decrease in non-mitochondrial respiration after *GDAP1* silencing in primary cells may indicate impaired peroxisomal function.

It is unclear if the AMPK-mediated reduction in *GDAP1* is adaptive. A meta-analysis of exercise-responsive genes in humans indicates that *GDAP1* is not significantly decreased due to exercise [207]. The discovery that AMPK reduces *GDAP1* expression in mice and in primary skeletal muscle cells is not in direct conflict with these results, since exercise *per se* involves various metabolic and physiological changes above and beyond AMPK activation. It could be that AMPK activation in isolation causes a decrease in *GDAP1* while the myriad other signals induced during exercise override this effect. In this research, *GDAP1* levels were not affected by exercise in healthy individuals, but were reduced after exercise in T2D patients. This bifurcation based on disease status may due to intact AMPK signaling in T2D [121] whereas other aspects of their metabolic function are disrupted.

GDAP1 silencing and AMPK activation affected circadian genes. A role for AMPK in in this context has been described [208], but this is the first report implicating *GDAP1*. Further study is needed to resolve the mechanism by which *GDAP1* affects core clock machinery.

4.4 NOVEL EFFECTS OF EXERCISE OR AMPK ACTIVITY IN HUMAN SKELETAL MUSCLE- REVISITING THE THESIS AIMS

The central theory of this thesis work has been that exercise drives beneficial metabolic changes in skeletal muscle, in large part due to AMPK activation. To this end, the research presented in this thesis demonstrates that:

1. Combining fasting with exercise enhances AMPK activation and the capacity to oxidize lipids in addition to altering the DNA methylation of key genes involved in metabolic adaptation to exercise.
2. Although it is modulated by insulin in other models, insulin stimulation does not alter FAK phosphorylation in human skeletal muscle. FAK phosphorylation is, however, reduced when AMPK is activated. Furthermore, FAK modulates lipid and carbohydrate handling in human skeletal muscle.
3. AMPK activity inhibits *GDAP1* expression in skeletal muscle. While GDAP1 plays a role in mitochondrial function in nerve cells, its role in human skeletal muscle is apparently non-mitochondrial. Nonetheless, GDAP1 does play a role in lipid oxidation, non-mitochondrial respiration, and modulating circadian gene expression.

This thesis has identified two novel targets of AMPK in human skeletal muscle, FAK and *GDAP1* gene expression. Additionally, overlaying fasting with exercise leads to increased AMPK activation and increased adaptive responses to the exercise.

The work in this thesis focuses on human skeletal muscle tissue, though exercise affects nearly every organ system of the body and AMPK plays a role in maintaining energy homeostasis in nearly all cells. A complete picture of how exercise and fasting interact to

influence whole-body physiology cannot be developed without future research. Specifically, how these lifestyle modifications impact AMPK signaling in liver, brain, adipose, and other tissues is required. The research in this thesis provides valuable discoveries that help to clarify the physiological and molecular mechanisms responsible for exercise- and AMPK-mediated metabolic adaptability.

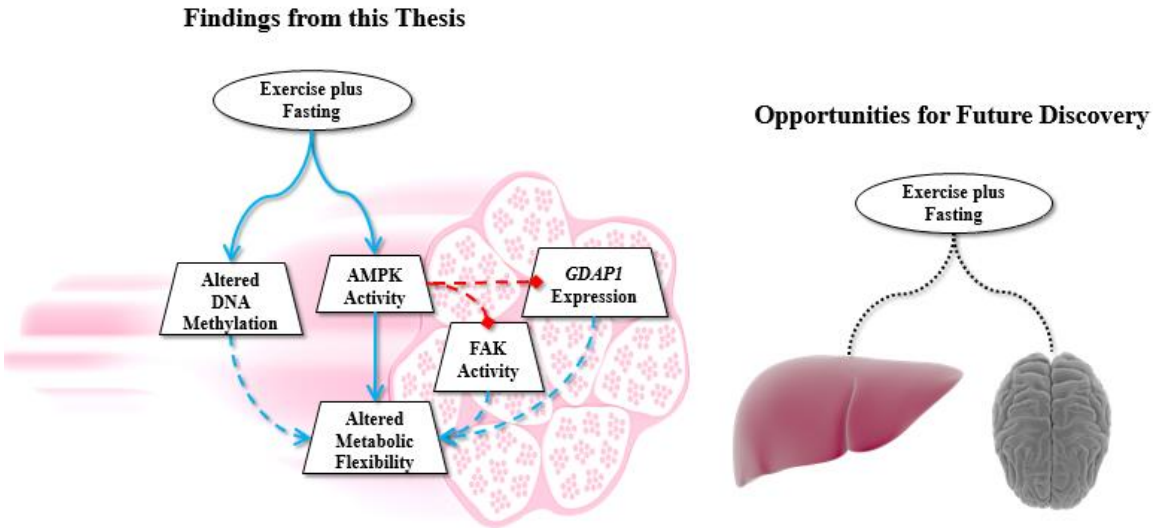

Figure 7:

Novel Effects of Exercise or AMPK Activation in Human Skeletal Muscle

Blue arrows indicate activating effects and red diamonds indicate inhibiting effects.
Solid lines indicate observed effects and dashed lines indicate plausible effects.
Black dotted lines indicate relationships to be investigated in future research.

5 CONCLUSION

When individuals adopt lifestyle modifications, including diet and exercise, health outcomes can be as good as pharmacological interventions in preventing progression to T2D [209, 210]. Although exercise as a prescription for good health has a long history, incidences of disorders responsive to exercise therapy continue to rise [1, 2]. Although the medical community has identified many symptoms that are improved due to exercise training, the specific mechanisms responsible for the improvements are incompletely resolved.

The research in this thesis compliments previous work regarding the benefits of engaging in sequential bouts of exercise without nutritional replenishment between. However, this thesis pushes the field forward by revealing that DNA methylation is also impacted by the diet and exercise protocol used. The papers in this thesis also establish that, despite the presence of a vast amount of previous work dissecting the role of AMPK in skeletal muscle, more discoveries are left to be made. Specifically, FAK phosphorylation and *GDAP1* expression are modulated by AMPK activity in skeletal muscle, and silencing either gene alters lipid metabolism.

To avoid misleading consumers of scientific literature, it is important to recognize and highlight the limitations of any given research model. The bulk of the research presented in this thesis is novel in that the roles of FAK and GDAP1 in human skeletal muscle had been previously undescribed. The previous research that had been done was only performed in simplified models (i.e., rodents or immortalized cell systems). Unfortunately, the results from the simplified models did not perfectly predict how these proteins behave in human skeletal muscle. This thesis provides data more directly relevant to human physiology.

In addition to physiological research, breakthroughs in the social sciences are needed to address the burgeoning problem of metabolic disorders. Even though lifestyle interventions, when followed, are effective, most individuals who undertake lifestyle interventions revert to old habits, and ultimately regain most of the original weight [211, 212]. Various psychological and social barriers prevent would-be beneficiaries from reaping the rewards of lifestyle changes [213]. With the advent and popularization of behavioral economics, the field of economics has shifted from presuming that individuals act in logical, self-interested, rational manners to recognizing that human behavior is not so simply described [214]. Similarly, it is time for the medical community to recognize that simple exercise prescriptions will not suffice —no matter how logical, self-interested, or rational patients may be. Both physiological research into the mechanisms responsible for exercise's beneficial effects, and social science research into motivation and decision-making, are needed to address the burden of metabolic disease. Global metabolic health in the 21st century is an interdisciplinary problem that necessitates interdisciplinary solutions.

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